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The Immobilisation of Bovine Serum Albumin, Acid Phosphatase, Glucose Oxidase and Phenyl Propylamine to Maleic Anhydride Block Copolymers

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SUMMARY

Maleic anhydride-based block copolymers have been prepared by various routes. These copolymers provide the basis for enzyme immobilisation without the need for secondary coupling agents. It is felt that problems arising from enzyme-support interactions are much reduced relative to systems using poly(acrylic acid)-branch supports. There are problems in stability in that these supports cannot be used above a pH of 7.4. At low pH values, gel formation arises. Such gel formation enhances the immobilisation efficiency considerably but generates media which are difficult to process. Ease of coupling was a feature of non-aqueous systems, suggesting that products obtained in this way should be of use in affinity separations.

INTRODUCTION

Block copolymers have been quite widely used as enzyme supports. However, these copolymers generally require activation with another reagent which tends to activate relatively few of the total number of the groups which are normally available for coupling to proteins. In addition, we have shown that where there is an excess of carboxylic acid groups on the copolymer branches of graft copolymers, a considerable reduction in the enzymic activity can occur because of interaction of the enzyme with these carboxylic acid groups (ABDEL-HEY et al, 1983). Block copolymers based on maleic anhydride-co-monomer composites offer a solution which has considerable appeal. These should react directly with amino groups. Also, when block copolymers containing maleic anhydride are used, advantage can be taken of the degree of relative alternation which arises during copolymerisation (DODGSON and EBDON, 1975; HESEDING and SCHNEIDER, 1977). This alternation causes the carboxylic groups, arising as a result of hydrolysis of the anhydride units during the coupling reaction, to occur with reduced frequency. Thus the possibility of problems arising from excessive interaction between the enzyme and carboxylic groups is reduced.

Another potential advantage of using a maleic anhydride block copolymer is the possible control of the hydrophobic/hydrophilic characteristics of the copolymer. This appears to have an effect on enzyme stability (ABDEL-HEY et al, 1983). The influence of the resulting hydrophilic carboxyl groups from the maleic anhydride could be modified by the degree of hydrophobicity of the co-monomer and by the frequency with which such

hydrophobicity occurs in the polymeric chain.

MANECKE et al (1977) immobilised 348-442mg of papain g^{-1} of copolymer of maleic anhydride/vinyl pyrrolidene cross-linked with divinylbenzene. These yields were excellent. Generally the cross-linked copolymers were gels after reaction; the retention of enzymic activity was 8-12%. GOLDSTEIN et al (1971) used a variety of maleic anhydride block copolymers, including ethylene, styrene and isobutylvinyl ether as the co-monomer, to immobilise naringinase. No indication was given as to the amount of protein immobilised but the retention of activity was shown to be very good. This is different from the results with alkaline phosphatase when only 1% of the enzymic activity was retained when the enzyme was coupled to block copolymers of maleic anhydride with polyethylene and methyl vinyl ether (ZINGARO and UZIELLE, 1970).

EXPERIMENTAL

A. Materials

Acid phosphatase, glucose oxidase, trypsin, bovine serum albumin (BSA) and 1,6 diaminohexane were obtained from Sigma Biochemicals Ltd, Missouri, USA. Styrene, acrylonitrile, methyl methacrylate, benzoyl peroxide and azo-bis-isobutyronitrile were obtained from the Aldrich Chemical Company, Gillingham, UK. The monomers were purified by vacuum distillation after removal of inhibitor. Vinyl acetate, maleic anhydride and all other reagents (analytical grade) were obtained from BDH Ltd, Poole, Dorset, UK. The vinyl acetate was purified by distillation.

B. Polymerisation Procedure

(i) Using Chemical Initiation (TURNEY and LAVIN, 1945), BLACKELEY and HELVELLE, 1956)

Maleic anhydride (30mmol) and the other monomer (styrene, vinyl acetate, methyl methacrylate or acrylonitrile) (10mmol) were dissolved in toluene ($200cm^3$). Benzoyl peroxide or azo-bis-isobutyronitrile (90mg) was added and the mixture was heated at 363K on an oil bath for 2 hours. The mixture was then added to petroleum ether (60-80°C) ($400cm^3$). The solids were filtered, washed with benzene, petroleum ether and then dissolved in acetone. The solids were re-precipitated by pouring into a ten times excess of water, filtered and dried 'in vacuo' at 313K.

(ii) Using γ-irradiation

The solutions of the monomers were as before but in chloroform ($30cm^3$). These solutions were irradiated in the presence of air at 2.5 $rad.s^{-1}$ for 24h using Co(60), γ irradiation. The resulting solids were filtered, dissolved in acetone and re-precipitated in petroleum ether (60-80°C), filtered and dried 'in vacuo' at 313K. The maleic anhydride content was determined by the method of LEVIN at al (1964) and the total maleic content by titration with 0.1M NaOH after total hydrolysis by heating in 0.5M NaCl for 30 minutes at 373K.

C. Coupling to proteins

Portions (100mg) of the block copolymers were shaken with protein (BSA or acid phosphatase or glucose oxidase or trypsin) (20mg) in water for 18 hours at 277K before filtering and washing with water. The supernatant and washings were combined and made up to 100cm^3 with distilled water and the protein content determined using the coomassie blue method (SEDMARK and GROSSBERG, 1977). The protein bound to each copolymer was calaculated as the difference between the result obtained with the copolymer present and a copolymer-free control. With the immobilised enzymes, the acid phosphatase activity was determined by the method of BESSY et al, (1966), the glucose activity by the method of HUGGETT and NIXON (1957) and the trypsin activity by the method of ERLANGER et al (1961).

D. Coupling to phenylpropylamine

Portions (100mg) of the block copolymers were stirred at room temperature with phenylpropylamine (40mg) in petroleum ether (10cm^3) for 16 hours and then filtered and washed with petroleum ether. The supernatant liquor and washings were made up to 50cm^3 with petroleum ether and the phenylpropylamine concentration was calculated from the difference between these block copolymer solutions and the copolymer-free control by measuring the absorbance at 260nm.

RESULTS AND DISCUSSION

As the hydrophobic/hydrophilic properties of the block copolymer would be affected by the nature of the co-monomer, four different co-monomers were selected for copolymerisation. The co-monomers will also influence the amount of maleic anhydride included in the co-polymer. The co-monomers selected were vinylacetate, styrene, acrylonitrile and methyl methacrylate.

Attempts to prepare the block copolymers using chemical initiation using benzoyl peroxide and 2-azo-bis-iso-butyronitrile failed to give any products with the exception of methyl methacrylate/maleic anhydride with benzoyl peroxide. Consequently Co(60) irradiation was used to initiate each system; all four co-monomers gave block copolymers. The yields are given in Table 1 and are reflective of the reactivity of the co-monomers. The total maleic acid and anhydride contents were determined by acid-base titration and the anhydride content was determined in anhydrous dimethylformamide using sodium methoxide. Some hydrolysis of the anhydride can occur during the polymer preparation. The maleic to co-monomer ratios were calculated and are given in Table 1. Under the best conditions a 1:1 ratio might be achieved. Three of the copolymers were in the range 1.9-2.8 to 1.

TABLE 1

Co-monomer	Yield g	Yield %	Concentration of constituents (mM) Anhydride	Acid	Comonomer	Ratio Maleic: Comonomer
Styrene	7.32	59	2.48	0.90	6.3	1:1.9
Vinylacetate	2.15	15	2.29	1.10	7.7	1:2.0
Methyl methacrylate	2.14	17	2.28	0.36	7.4	1:2.8
Acrylo-nitrile	2.00	67	0.31	0.13	18.0	1:41

Table 1 The yield and composition of a number of maleic anhydride containing block copolymers obtained by the Co(60) ,irradiation of chloroform solutions of the monomers.

Residues are well dispersed along the copolymer branch chain and this could have a pronounced effect on the retention of enzymic activity if the copolymer is sufficiently reactive. The lower concentration of anhydride could be due to a relatively higher reactivity of the acrylonitrile in solution.

The chemically initiated block copolymers of maleic anhydride and methyl methacrylate contained 3.3mmol anhydride, 0.5mmol of acid and 6.14mmol of co-monomer. This gave a ratio of 1:1.6 which indicated that the frequency of anhydride groups was greater than for the Co(60)-initiated block copolymer which had a ratio of 1:2.8.

It is possible that the frequency of the anhydride residues could be adjusted by using different ratios of monomers. However, it was thought to be of greater value to determine how effective the copolymers were in the immobilisation of proteins.

A study of all of the block copolymers showed that they were soluble above a pH of 7.4, due to the formation of the sodium salt of the acid. Re-precipitation with acid gave gels.

Protein Coupling

Portions of each of the block copolymers were treated with solutions of bovine serum albumin in water at 277K for 18 hours. The yields of coupling, defined as the amount of protein (mg) immobilised g^{-1} of each copolymer are given in Table 2. The vinyl acetate copolymer formed a gel and gave excellent yields of coupling. The level obtained was much greater than for maleic anhydride-containing graft copolymers (BEDDOWS et al 1985a, 1985b).

TABLE 2

Co-monomer	Protein Immobilised				
	BSA		Acid Phosphatase		
	Coupled (mg g^{-1})	Absorbed* (mg g^{-1})	Coupled (mg g^{-1})	Absorbed* (mg g^{-1})	Active Enzyme immobilised (mg g^{-1})
Styrene	21.0	0.0	32	0.09	0.0
Vinyl Acetate	445.0	17.0	188	52	15
Methyl methacrylate	77.5	0.0	0	0	0
Acrylonitrile	48.0	0.0	83	3.1	5.7

Table 2 Immobilisation of BSA and Acid Phosphatase onto maleic anhydride - containing block copolymers at 277K in water after 18 hours

* amount absorbed determined with fully hydrolysed block copolymer.

The other three copolymers did not give gels although they had a tendency to absorb water. The lack of gel formation could explain the lower yields of coupling obtained with these systems and why no protein was absorbed. The styrene-containing copolymer gave the lowest level and this could be due to its strongly hydrophobic nature; the styrene could have interacted with itself during the re-precipitation stage to give hydrophobic portions on the chain which could discourage the approach of the protein.

The amount of protein absorbed was determined using the hydrolysed block copolymer. This can only be regarded as an approximation since the carboxyl groups formed on hydrolysis would undoubtedly cause a conformation of the block copolymer and would also influence the approach of the protein.

The rate of hydrolysis of the methyl methacrylate in water showed that it was slow to hydrolyse and that a significant anhydride content was still present after 18 hours, whereas the styrene block copolymer was fully hydrolysed after 6 hours.

Portions of each block copolymer were used to immobilise acid phosphatase at 277K over 18 hours. The results are given in Table 2 and show that the vinyl acetate-containing copolymer absorbed a large amount of enzyme. However, it formed a gel but the amount of enzyme immobilised was very appreciable. The other copolymers did not form gels.

The activity of the immobilised enzyme was calculated in terms of residual

fully-active enzyme. Only the vinyl acetate and the acrylonitrile copolymers had any activity. The gel-forming characteristics of the vinyl acetate/maleic anhydride copolymer would limit its use. Surprisingly, the methyl methacrylate copolymer did not immobilise or absorb any enzyme.

As LEVIN et al (1964) immobilised 46-700mg of trypsin g^{-1} of maleic anhydride-ethylene block copolymers and reported a retention of 48-53% of the enzymic activity, attempts were made to immobilise trypsin to the block copolymers. However, only negligible amounts of protein were immobilised. This is difficult to explain.

Consequently, the most insoluble of the copolymers, i.e. the acrylonitrile/maleic anhydride copolymer was used to immobilise glucose oxidase. A yield of 39.25mg of enzyme g^{-1} of copolymer was obtained but the activity, equivalent to 1.54mg of active enzyme g^{-1} of copolymer, represented a retention of only 4% of the activity.

With the exception of the vinyl acetate-co-maleic anhydride copolymer the results are somewhat disappointing compared to those obtained by MANECKE (1979). However, he reported having used a cross-linking agent which would tend to give a more open and stable hydrophobic structure. Consequently, the vinyl acetate and the acrylonitrile block copolymers were treated with 1,6-diaminohexane according to the method of LEVIN et al (1964). The diamine could cross-link in the 5 minute period prior to the introduction of the protein and still be hydrophilic as only a few of the diaminohexane molecules will have the chance to link through both the amino groups. When BSA was introduced, both copolymers showed a decreased yield of immobilisation; the vinyl acetate/maleic anhydride still gave a gel which immobilised 65mg of BSA/g of copolymer and the acrylonitrile/maleic anhydride copolymer immobilised 26.3 mg/g. The latter was not a gel and so was used to immobilise acid phosphatase. However, although 36mg of enzyme was immobilised g^{-1} of copolymer the retention of activity was not significantly greater than without the diaminohexane (2.1mg of active enzyme g^{-1} of copolymer).

Coupling to phenylpropylamine

As affinity chromatography systems generally use small molecules linked through the amino group, the block copolymers were used to couple to phenylpropylamine as non-aqueous conditions could be used and the loss of copolymer due to hydrolysis would be greatly reduced. The results (Table 3) showed that 42-68% of the available anhydride groups had coupled to the amine.

TABLE 3

Co-monomer	Phenyl Propylamine (mg/g copolymer)	Theoretical value	% of groups reacted
Vinyl acetate	129	310	42
Styrene	233	344	68
Methyl methacrylate	207	310	67

Table 3 Coupling of phenylpropylamine to various maleic anhydride containing block copolymers

CONCLUSIONS

The use of maleic anhydride-containing block copolymers, with vinyl acetate, styrene and acrylonitrile as the co-monomer, are of limited value in that they cannot be used above pH 7.4 and below this, the formation of a gel appears to enhance the immobilising ability with proteins greatly. It would appear that the introduction of more hydrophobic cross-linking reagents would be beneficial and this is being investigated further.

In a non-aqueous system, the copolymers were very effective in coupling to phenylpropylamine and could be of use in affinity systems.

REFERENCES

ABDEL-HEY, FI, BEDDOWS, CG, GIL, FH, GUTHRIE, JT, J Polymer Sci., 21, 2463-2472 (1983).

BEDDOWS, CG, GIL, MH, GUTHRIE, JT, Biotech. Bioeng., 27, 579-584 (1985).

BEDDOWS, CG, GIL, MH, GUTHRIE, JT, Biotech. Bioeng., (in press 1085).

BLACKELEY, DC, HELVILLE, HW, Makromol. Chemie., 18, 20 (1956).

BESSEY, PA, LOWRY, OH, BROOK, MJ, Biol. Chem., 164, 321 (1946).

DODGSON, K, EBDON, J, Europ. Polym. J., 13, 791 (1977).

ERLANGER, BF, KOKOWSKY, N, COHEN, W, Arch. Biochem. Biophys., 95, 271 (1961).

GOLDSTEIN, L, LIFSHITZ, A, SOKOLOVSKY, M, Int. J. Biochem., 2, 448 (1971).

HESEDING, C, SCHNEIDER, C, Europ. Polym. J., 13, 387 (1977).

HUGGETT, AG, NIXON, DA, Lancet, 2, 368 (1957).

LEVIN, Y, PECHT, M, GOLDSTEIN, L, KATCHALSKI, E, Biochemistry, 3, 1905 (1964).

MANECKE, G, KOREWZECKER, R, Makromol. Chem., 178, 729 (1977).

SEDMARK, JJ, GROSSBERG, SE, Anal. Biochem., 79, 544 (1977).

TURNEY, A, and LAVIN, E, J. Amer. Chem. Soc., 67, 2044 (1945).

ZINGARO, RA, USIELL, M, Biochem. Biophys. Acta., 213, 371 (1970).

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